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Chemokine induction through Toll-like receptor 3 in human dental pulp cells

사람치수세포에서 Toll 유사 수용체 3을 통한 케모카인 유도

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하예은

ABSTRACT

Chemokine induction through Toll-like receptor 3 in human dental pulp cells

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Objectives

Human dental pulp cells (human DPCs) in pulp cavity have mesenchymal stem cell properties and are capable of differentiating into odontoblasts, adipocytes, chondrocytes, osteoblasts and neurons. Human DPCs, once exposed to microorganisms in the oral cavity by dental caries, dental trauma and dentinal tubule exposure, which in turn trigger inflammatory responses against microbe-associated molecular patterns (MAMPs) resulting pulpitis. During the development of pulpitis, inflammatory mediators including IL-1, IL-6, IL-8, TNF-α and MMP-9 contribute to pathogenesis. In the oral cavity, various viruses exist and can lead to several infectious diseases. When pulp tissues are exposed to oral microorganisms, some

oral viruses can stimulate DPCs likely using their MAMPs. However, the understanding immune responses of human DPCs induced by viral infection is limited. In this study, the immunological properties of human DPCs against viral double-stranded RNA (dsRNA) were investigated.

Methods

All experiments using human DPCs were conducted under the approval of the Institutional Review Board of the Seoul National University (S-C20200023). To determine the expression of pattern-recognition receptors (PRRs) which are essential for the recognition of MAMPs, the mRNA expression of Toll-like receptors (TLRs) in human DPCs was analyzed by real-time RT-PCR. In this study, synthetic poly(I:C), mimicking viral dsRNA, was used as a model for RNA viral infection. To investigate the expression of poly(I:C)-induced inflammatory cytokines, the expressions of IL-8, CXCL10, CCL2, CCL5, CCL20, IFN-β, IL1-β, IL-6 and TNF-α were analyzed by real-time RT-PCR. In addition, the productions of IL-8, CXCL10, CCL2, IL-1β, IL-6 and TNF-α were measured by ELSIA. To knock down TLR3, human DPCs were transfected with TLR3 siRNA, and subsequently, stimulated with poly(I:C) followed by determination of the expression of IL-8 with real-time RT-PCR and ELISA. To elucidate the signaling pathway activated by poly(I:C), human DPCs were pre-treated with MAP kinase inhibitors, SB203580, SB202190, PD90859, ERK inhibitor II, SP600125 or JNK inhibitor II, for 1 h, and then stimulated with poly(I:C) for additional 24 h. To determine which transcriptional factors are essential for poly(I:C)-induced IL-8 expression in human DPCs, the cells were transfected with luciferase reporter gene of which the expression is regulated by human IL-8 promoter (-132/+42)/pGL3 wild type (WT), mutant (mut) AP-1, mut C/EBPβ and mut NF-κB, respectively, and then the promoter activity induced by poly(I:C) was analyzed by luciferase assay.

Results

Among TLRs (1-10) tested, TLR3 was the most highly expressed in human DPCs. Poly(I:C) potently induced IL-8 expression in a time- and dose-dependent manner. Besides, the expressions of CXCL10, CCL2, CCL5, CCL20, IFN-β and IL-6 were substantially increased by poly(I:C) in human DPCs while IL1-β and TNF-α were not detected. To determine whether the expression of poly(I:C)-induced inflammatory cytokines is dependent on TLR3, human DPCs were transfected with TLR3 siRNA and then stimulated with poly(I:C). human DPCs transfected with TLR3 siRNA showed a decreased IL-8 production compared with non-targeting siRNA-transfected cells. In addition, the expressions of cytoplasmic dsRNA receptors, RIG-1 and MDA5, were significantly increased by poly(I:C) stimulation. To investigate the signaling pathway involved in poly(I:C)-induced cytokine production, human DPCs were pre-treated with p38 inhibitors (SB203580 and SB212190), ERK inhibitors (PD90859 and ERK inhibitor II) or JNK inhibitor (SP600125 and JNK inhibitor II), respectively. As a result, poly(I:C)-induced IL-8 production was substantially decreased by p38, ERK or JNK inhibitors in a dose dependent-manner, suggesting that poly(I:C)-induced IL-8 production is mediated by MAP kinase signaling pathway. Indeed, C/EBPβ and NF-κB are involved in poly(I:C)-induced IL-8 expression in human DPCs. Furthermore, synergistic effect of poly(I:C) and bacterial components or metabolites were tested considering that subsequencial invasion of viruses or bacteria can worse the periodontal disease symptoms. A synthetic lipopeptide Pam2CSK4 mimicking bacterial lipoproteins showed synergistic effect with poly(I:C) in IL-8 production in human DPCs. In contrast, short chain fatty acids (SCFAs) such as butyrate, propionate and acetate, produced by microbiota metabolism, potently suppressed poly(I:C)-induced IL-8 secretion in order.

Conclusions

Collectively, the current study demonstrated that TLR3 is the most highly expressed on human DPCs among TLRs 1-10 and poly(I:C) is a potent immune stimulator which activates TLR3 signaling pathway. In addition, TLR2 ligand, such as Pam2CSK4, potentiates poly(I:C)-induced inflammatory responses in human DPCs.

On the other hand, SCFAs, microbial metabolites, down-regulate poly(I:C)-induced chemokine production. This study will provide new insight of innate immune responses by viral infection and viral-bacterial interactions in pulpal microenvironment.

Keywords: Dental pulp cells, Double-stranded RNA, Poly(I:C), TLR3, IL-8

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CONTENTS

Chapter I. Introduction

The oral cavity provides dynamic environment for supporting growth of microorganisms and is a space where host cell and microorganisms coexist. The oral cavity consists of the soft and the hard tissues in which form heterogeneous ecological systems and diverse microbial communities [1]. The oral microbiome contains bacteria, viruses, fungi, protozoa and archaea [2]. To date, more than 1000 species of microbial species have been detected in the oral cavity and oral microbiota interact with various host cells [3, 4]. Host-microbe interaction is essential for maintaining immunological homeostasis. Human microbiota provides beneficial functions including maturation of host mucosa, metabolic regulation, immune system development and regulation of inflammatory response [3]. However, imbalance between host and microbiota can contribute to infectious diseases. For instance, dysbiosis of oral microbiota can lead to periodontal diseases including gingivitis, periodontitis and apical periodontitis [5, 6]. In particular, viral infection is closely related with various oral infectious diseases. It is reported that *Herpesviruses* are frequently isolated in the oral cavity of patients with periodontitis [7]. Also, *Papillomaviruses, Picornaviruses* and *Retroviruses* are associated with oral diseases such as periodontitis and gingivitis [8]. Furthermore, Herpes simplex virus exacerbates oral diseases such as oral Kaposi's sarcoma, hairy leukoplakia and oral candidiasis [9].

Innate immunity is essential for initiating immune responses against various microbial interactions. Host cells have pattern recognition receptors (PRRs) for

sensing microbe-associated molecular patterns (MAMPs). Toll-like receptor (TLR) is a family of typical PRR. TLR1, TLR2, TLR4 and TLR5 are mainly expressed on the host cell surface and recognize bacterial components. TLR2 recognizes lipopeptide [10], lipoproteins [11] and lipoteichoic acids [12]. TLR4 and TLR5 recognize lipopolysaccharide (LPS) [13] and flagellin, respectively [14]. Whereas, TLR3, TLR7, TLR8 and TLR9 are mainly located in endosome and recognize viral or bacterial nucleic acids [15]. TLR3 recognizes endosomal double-stranded RNA (dsRNA) [16], TLR7 and TLR8 recognize single-stranded RNA (ssRNA), and TLR9 recognizes CpG DNA [17]. Subsequently, interaction between TLRs and MAMPs activates their adaptor molecules and signaling pathways leading to pro-inflammatory responses. Especially, recognition of nucleic acids from bacteria or viruses is crucial for defense against bacterial or viral infections. TLR3, cytoplasmic RIG-1 and MDA5 sense dsRNA and induce production of type I IFNs and pro-inflammatory cytokines (Figure 1) [18, 19]. The recognition of viral dsRNA by TLR3 induces TRIF-mediated signaling pathway and IRF3 activation leading to IFN-β production which inhibits viral replication [20, 21]. In addition, TLR3 activation leads to MAPK signaling and NF-κB activation to produce pro-inflammatory cytokines [16, 22].

Dental pulp is enveloped in enamel and dentin. In the pulp cavity, there are immunocompetent dental pulp cells and they trigger inflammatory responses against MAMPs when exposed to oral microorganisms (Figure 2) [23-25]. Pulpitis is mainly caused by microbial infection resulting from dental caries, dentinal tubule exposure, and dental trauma [26, 27]. In the development of pulpitis, inflammatory mediators

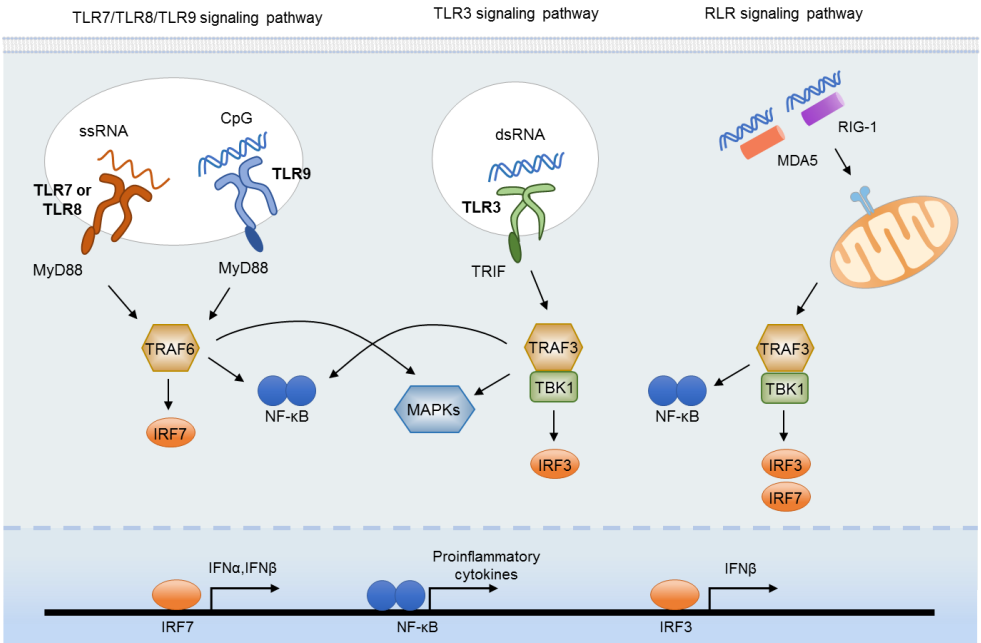

Figure 1. Nucleic acid sensors and signaling pathways. Endosomal TLR7 and TLR8 recognize ssRNA, and TLR9 recognizes CpG DNA. TLR7, TLR8 and TLR9 induce MyD88-mediated signaling which activates IRF7, NF-κB and MAPK signaling. The recognition of dsRNA by endosomal TLR3 induces TRIF-mediated signaling pathway which activates IRF3 leading to IFN-β production. The TLR3–TRIF signaling pathway also activates NF-κB and MAPKs and results in the production of pro-inflammatory cytokines. Also, the cytoplasmic dsRNA receptors RIG-I and MDA5 activate mitochondria-associated adaptor molecules and induce the production of type I IFNs and other cytokines by activation of IRF3, IRF7 and NF-κB. *Modified from Gilliet et al. Nature Reviews Immunology, 2008.*

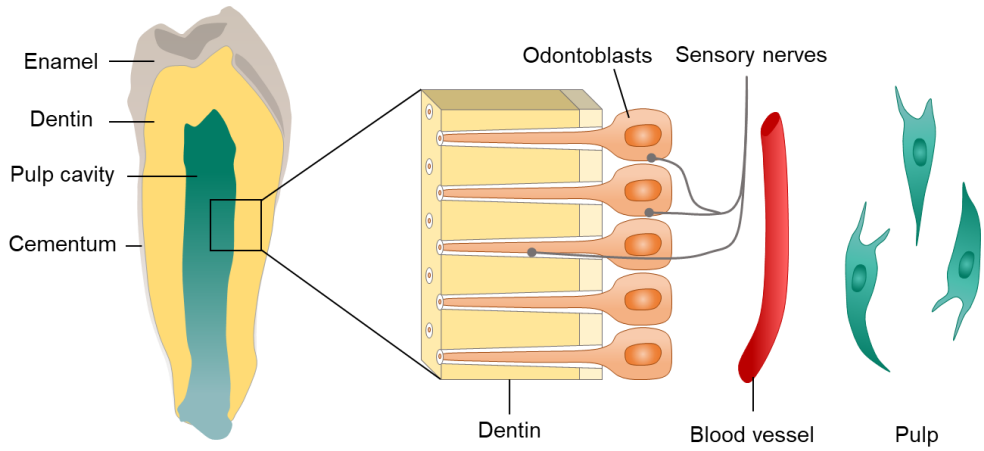

Figure 2. Structure of dentin-pulp complex. Dentin is a major component of tooth and surrounded by enamel and cementum. The pulp is located in the center of a tooth and contains nerves, blood vessels and immunocompetent cells. Odontoblasts are located in outermost layer of pulp and extend the processes to dentinal tubules.

including IL-1, IL-6, IL-8, TNF-α and MMP-9 contribute to pathogenesis [28, 29]. Accumulating studies have reported that IL-8 expression is potently up-regulated in the individuals with periodontal diseases [30, 31]. IL-8 is a potent chemokine for infiltration and activation of neutrophils, also, stimulate lymphocytes and monocytes to initiate innate and adaptive immune responses. [32, 33]

Human dental pulp cells (human DPCs) are isolated from pulp tissues and one of dental stem cell source [34]. Human DPCs express typical mesenchymal stem cell (MSC) markers such as CD44, CD146 and STRO-1 [35]. Indeed, DPCs are able to differentiate into odontogenic [34], adipogenic [36], chondrogenic [37], osteogenic [38] and neurogenic [39] lineages. Since human DPCs have self-renewal and multipotent properties, they play important roles in tissue regeneration upon injury [40]. Because human DPCs are essential for modulating immune responses [41], the effect of TLR ligands on immunological properties in human DPCs have been studied. Hirao *et al.* reported that in human DPCs, lipopeptide and LPS induces IL-8 production through TLR2 and TLR4 activation, respectively. [24]. Moreover, it is reported that TLR5 activation by bacterial flagellin induces production of urokinase plasminogen and IL-6 in human DPCs [42]. However, the understanding immune responses of human DPCs induced by viral infection is limited. In this study, the immunological properties of human DPCs against dsRNA were investigated.

Chapter II. Materials and Methods

2.1. Reagents and chemicals

Poly(I:C) (High molecular weight) and Pam2CSK4 were purchased from Invivogen (San Diego, CA, USA). PE-conjugated monoclonal antibody specific for human TLR3 and eBioscience™ Foxp3/Transcription factor staining buffer set were obtained from Invitrogen (Carlsbad, CA, USA). The inhibitors of p38 (SB203580, SB202190) and ERK (PD90859, ERK inhibitor II) were purchased from Calbiochem (San Diego, CA, USA). The inhibitors of JNK (SP600125, JNK inhibitor II) were purchased form Merck Millipore (Darmstadt, Germany) and Sigma-Aldrich (St. Louis, MO, USA). An NF-κB inhibitor was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phorbol 12-myristate 13-acetate (PMA) and short chain fatty acids (SCFAs; sodium acetate, sodium propionate and sodium butyrate) were purchased from Sigma-Aldrich.

2.2. Cell culture

All experiments using human DPCs were conducted under the approval of the Institutional Review Board of the Seoul National University (S-D20200023). Human DPCs were obtained as previously described [43]. In brief, the pulp tissues were removed from the human teeth, then minced, and cultured in Alpha modification of Eagle's minimum essential medium (Alpha-MEM; Welgene, Seoul, Korea) containing 10% heat-inactivated fetal bovine serum (FBS; Gibco, Burlington, ON, Canada), 100 U/ml penicillin, and 100 μg/ml streptomycin (Hyclone, Logan, UT, USA) at 37□ in a 5% CO_2 humidified incubator. THP-1 cells, a human monocytic

cell line, were obtained from the American Type Culture Collection (Mannas, VA, USA) and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37□ in a 5% CO_2-humidified incubator. The cells were differentiated into macrophages by treatment with 100 nM of PMA for 2 days. The culture medium was replaced with fresh culture medium, followed by an additional incubation for 24 h prior to stimulation with poly(I:C).

2.3. Enzyme-linked immunosorbent assay (ELISA)

Human DPCs were plated on a 96-well culture plate overnight and stimulated with indicated stimuli. IL-8, CXCL10, CCL2, IL-1β and IL-6 in the culture supernatants were measured by commercial ELISA kits (Biolegend, San Diego, CA, USA) according to the manufacturer's instructions. TNF-α ELISA kit was purchased from R&D systems (Minneapolis, MN, USA).

2.4. Real-time reverse transcription-polymerase chain reaction (Real-time RT-PCR)

Total RNA was isolated from human DPCs by TRIzol reagent (Thermo Fisher Scientific, Lafayette, CO, USA) according to the manufacturer's instruction. Complementary DNA was synthesized from total RNA with random hexamer and reverse transcriptase (Promega, Madison, WI, USA). Primer sequences used in this study are shown in Table 1. The relative mRNA expressions were normalized with GAPDH and assessed by $2^{-\Delta\Delta CT}$ method.

Table 1. Primer sequences used in this study

Gene		Sequence (5' → 3')
TLR1	F	GGGCACCCCTACAAAAGGAA
	R	TGGCAAAATGGAAGATGCTAGTC
TLR2	F	CTTCACTCAGGAGCAGCAAGC
	R	ACACCAGTGCTGTCCTGTGACA
TLR3	F	GCCATGAAGTTGCTGACTGC
	R	TGAAGTTGGCGGCTGGTAAT
TLR4	F	CAGGATGATGTCTGCCTCGC
	R	TTAGGAACCACCTCCACGCAG
TLR5	F	TGCTACTGACAACGTGGCTT
	R	CCAGGAAAGCTGGGCAACTA
TLR6	F	CCTTCTCTGTGCCTCACCTG
	R	AAAACAAAAGCGCGCATCCT
TLR7	F	CCTTGTGCGCCGTGTAAAAA
	R	GGGCACATGCTGAAGAGAGT
TLR8	F	TGCTGCAAGTTACGGAATGAA
	R	ACTGAAGTGTCAAGAGAACCAT
TLR9	F	GAAAAGGACAAGTCGGCAGC
	R	CCAGTAGCGGGTACACCTTG
TLR10	F	AACGAATCATCCACGCACCT
	R	CTGCTGAATTCCCACGGCTT
IL8	F	GTGAAGGTGCAGTTTTGCCA
	R	TCTCCACAACCCTCTGCAC
CXCL10	F	GATGTTCTGACCCTGCTTCA
	R	GAAAGAATTTGGGCCCCTTG
CCL2	F	TCCCCAGACACCCTGTTTTA
	R	CAAAACATCCCAGGGGTAGA
CCL5	F	GAAAGAACCGCCAAGTGTGT
	R	GTAGAATCTGGGCCCTTCAA
CCL20	F	GCCAATGAAGGTGTGACA
	R	AACCCCAGCAAGGTTCTTTC
IFNB	F	GACCAACAAGTGTCTCCTCCA
	R	GGCAGTATTCAAGCCTCCCA
IL1B	F	CAGAAGTACCTGAGCTCGCC
	R	AGATTCGTAGCTGGATGCCC
IL6	F	CTTCGGTCCAGTTGCCTTCT
	R	TGGAATCTTCTCCTGGGGGT
TNFA	F	AGCTGGTTATCTCTCAGCTC
	R	CAGGGACCTCTCTCTAATCA
DDX58	F	AGAGCACTTGTGGACGCTTT
	R	TGTTTTGCCACGTCCAGTCA
IFIH1	F	CGAAGCAAGCCAAAGCTGAA
	R	TGGCAAACTTCTTGCATGGC
GAPDH	F	AAGTGAAGGTCGGAGTCAA
	R	ATGACAAGCTTCCCGTTCTC

2.5. Flow cytometry analysis

Human DPCs were stained with PE-conjugated monoclonal antibodies specific for TLR3 (Invitrogen) for 30 min at 4□ and washed with PBS. To examine intracellular TLR3 expression, human DPCs were fixed and permeabilized with eBioscience™ Foxp3/Transcription factor staining buffer set (Invitrogen) according to manufacturer's instruction. The expression of TLR3 was analyzed with flow cytometry (FACSVerse, BD Biosciences, San Jose, CA, USA). The percentage of PE-positive subset was analyzed with FlowJo software (TreeStar, San Carlos, CA, USA).

2.6. Transfection with small interfering RNA (siRNA)

Human TLR2, TLR3 siRNA and non-targeting siRNA pool were purchased from Dharmacon (Lafayette, CO, USA). Human DPCs were plated on a 6-well plate and transfected with 25 nM of human TLR2 siRNA, TLR3 siRNA or non-targeting siRNA pool using Viromer GREEN transfection reagent (Lipocalyx, Halle, Germany) according to manufacturer's instruction and then incubated for 48 h. The transfected cells were plated and stimulated with 10 μg/ml of poly(I:C). The mRNA expression levels of IL-8 and IL-6 were analyzed by real-time RT-PCR and protein levels in the culture supernatants were measured by ELISA.

2.7. Luciferase assay

The luciferase reporter plasmid containing human IL-8 promoter (-132/+42) wild

type (WT), mutant (mut) AP-1, mut C/EBPβ and mut NF-κB mutated on each transcriptional factor binding site were kindly provided by Dr. Myung Hyun Sohn (Yonsei University, Seoul, Korea). The hIL-8 promoter fragment was cloned into pGL3 vector and details of plasmid construct were previously described [44]. Human DPCs were plated on a 6-well plate and transiently transfected with human IL-8 promoter/pGL3 and *Renilla* luciferase plasmid (pRL-TK) using Attractene transfection reagent (Qiagen, Germantown, MD, USA) for 24 h. The transfected human DPCs were stimulated with 10 μg/ml of poly(I:C) for 24 h. The cells were lysed and luciferase activity was measured by Bright-Glo luciferase assay system (Promega) and normalized with *Renilla* luciferase assay system (Promega).

2.8. Statistical analysis

All data are expressed as mean values ± standard deviations (S.D.) from triplicates unless otherwise stated. Treatment groups were compared with an appropriate control group, and statistical analysis was determined using *t*-test. Differences were considered significant when $P < 0.05$.

Chapter III. Results

3.1. TLR3 is highly expressed in human DPCs

To investigate the immunological properties of human DPCs against MAMPs, the expression of various TLRs in human DPCs from three donors was firstly measured by real-time RT-PCR. The relative mRNA expression of TLRs 1-10 was calculated comparing with TLR9 because mRNA expression of TLR9 is the lowest among detectable mRNA expression levels of TLRs 1-10 (Figure 3A-C). As a result, TLR3 mRNA expression was the highest among the TLRs. The relative mRNA expressions of TLR6, TLR5, TLR1 and TLR4 were highly expressed in order, however, TLR7 and TLR8 were negligibly detected. Furthermore, the protein expression of TLR3 in human DPCs from three donors was determined by flow cytometry. Human DPCs were stained with PE-conjugated anti-human TLR3 antibodies or its isotype control antibodies with or without permeabilization. The indicated percentages are PE-positive subsets of human DPCs. As shown in Figure 4A-C, TLR3 expression was mainly detected in intracellular compartment rather than surface and it varied with individuals.

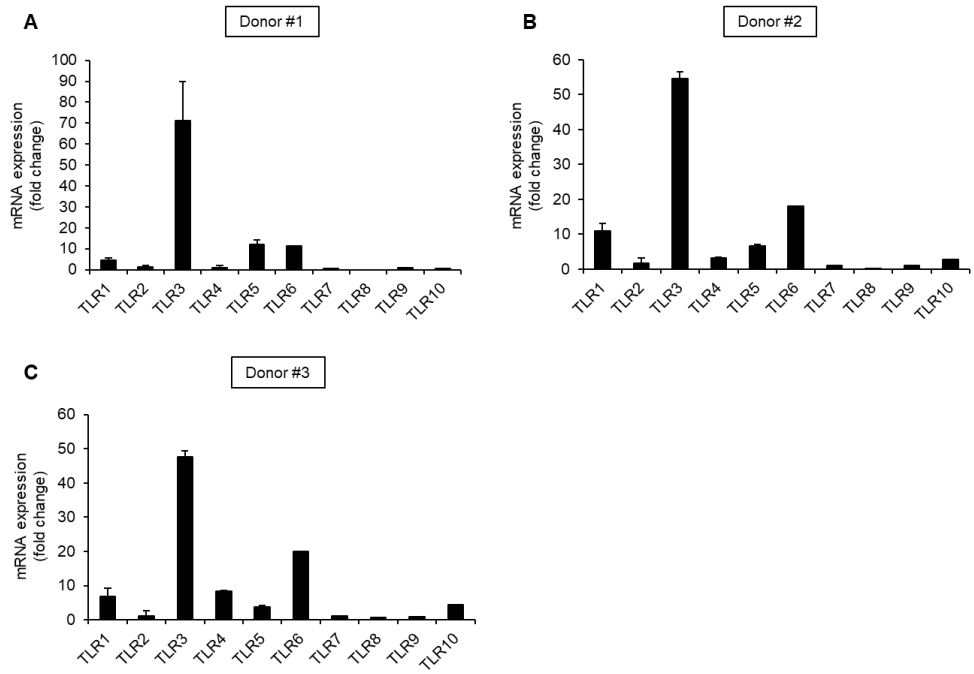

Figure 3. TLR3 is highly expressed in human DPCs. Human DPCs (1×10^5 cells/ml) were cultured in a 6-well plate for one day and then detached with Trypsin/EDTA. Total RNA was isolated from human DPCs. mRNA expression levels of TLRs 1-10 were determined by real-time PCR in human DPCs from donors #1 (A), #2 (B), and #3 (C). The relative mRNA expression of TLRs 1-10 was indicated as fold changes compared with TLR9 expression. All results are expressed as mean ± SD of triplicate samples.

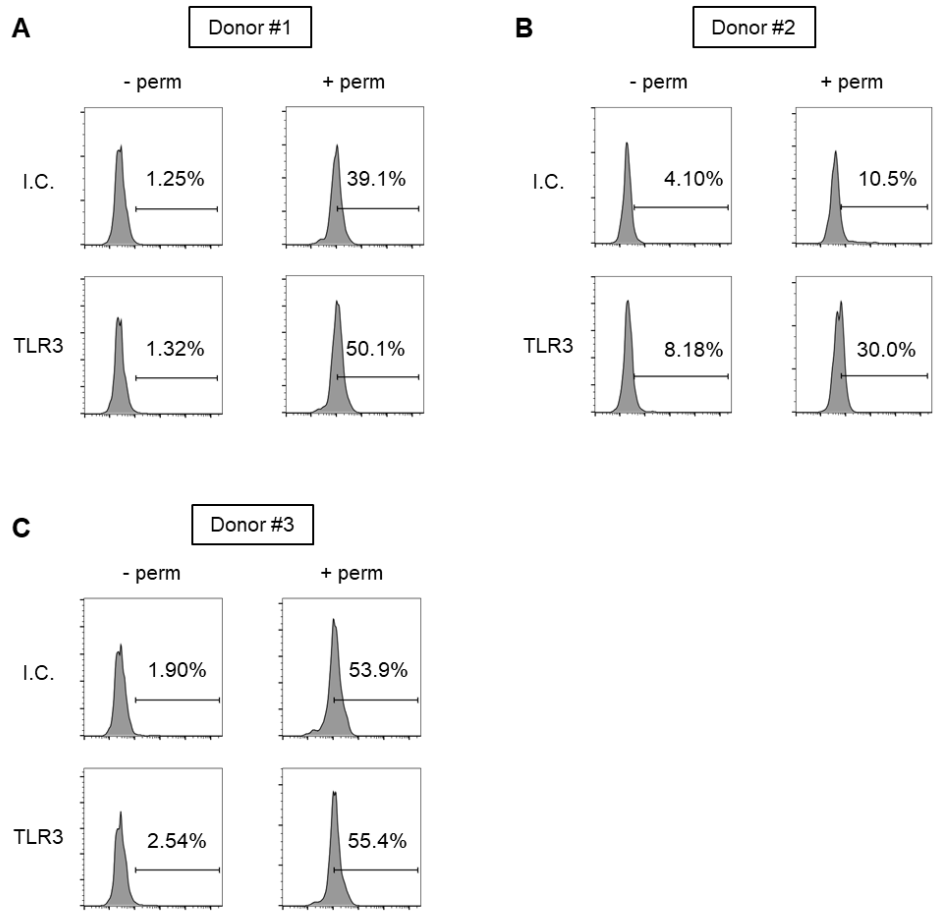

Figure 4. TLR3 expression is mainly detected in intracellular in human DPCs. Human DPCs (1×10^5 cells/ml) were stained with anti-human TLR3 or isotype control antibodies conjugated with PE. To determine intracellular expression of TLR3, human DPCs were fixed and permeabilized before intracellular antibody staining. The expression of TLR3 in human DPCs from donors #1 (A), #2 (B), and #3 (C) was analyzed by flow cytometry. The percentage of PE-positive subsets was indicated by range bar. –perm denotes non-permeablized human DPCs and + perm denotes permeabilized human DPCs. I.C. indicates isotype control.

3.2. Poly(I:C) induces the expression of pro-inflammatory cytokines and chemokines in human DPCs

To investigate how human DPCs initiate immune response against dsRNA, mRNA expressions of other cytokines and chemokines were assessed by real-time RT-PCR. Figure 5 showed that the mRNA expression levels of *IL8, CXCL10, CCL2, CCL5, CCL20, IFNB, IL1B, IL6* and *TNFA* were significantly increased by poly(I:C). When the cells were stimulated with 10 μg/ml of poly(I:C) for 3 h, *IL8* increased 768-fold, *CXCL10* increased 1467-fold, *CCL2* increased 67-fold, *CCL5* increased 51-fold, *CCL20* increased 32-fold, *IFNB* increased 81-fold, *IL1B* increased 4-fold, *IL6* increased 195-fold and *TNFA* increased 844-fold. Next, the protein levels of poly(I:C)-induced cytokines and chemokines in the culture supernatants were determined by ELISA. As a result, IL-8, CXCL10, CCL2 and IL-6 secretions were significantly increased in the presence of poly(I:C) (Figure 6A-D). However, IL-1β and TNF-α were rarely detected from human DPCs (Figure 6E-F). Pam3CSK4-treated THP-1 cells were used as a positive control for assay.

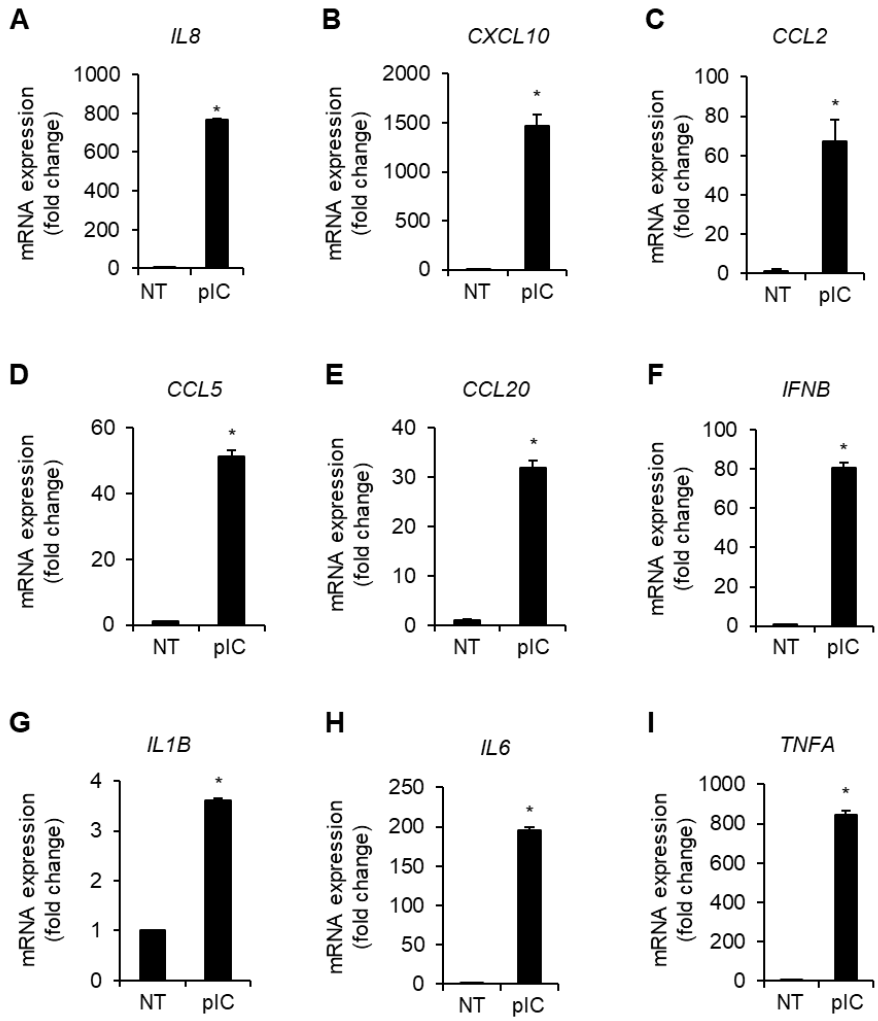

Figure 5. Poly(I:C) induces gene expression of pro-inflammatory cytokines and chemokines in human DPCs. (A-I) Human DPCs were stimulated with 10 μg/ml of poly(I:C) for 3 h. Total RNA was isolated from human DPCs (1×10^5 cells/ml). mRNA expressions of indicated cytokines and chemokines were determined by real-time PCR. NT denotes non-treatment and pIC denotes poly(I:C). The results shown are representative of triplicated experiments. All results are expressed as mean ± SD of triplicate samples. Asterisks (*) indicate statistically significant differences ($P < 0.05$) compared with appropriate controls.

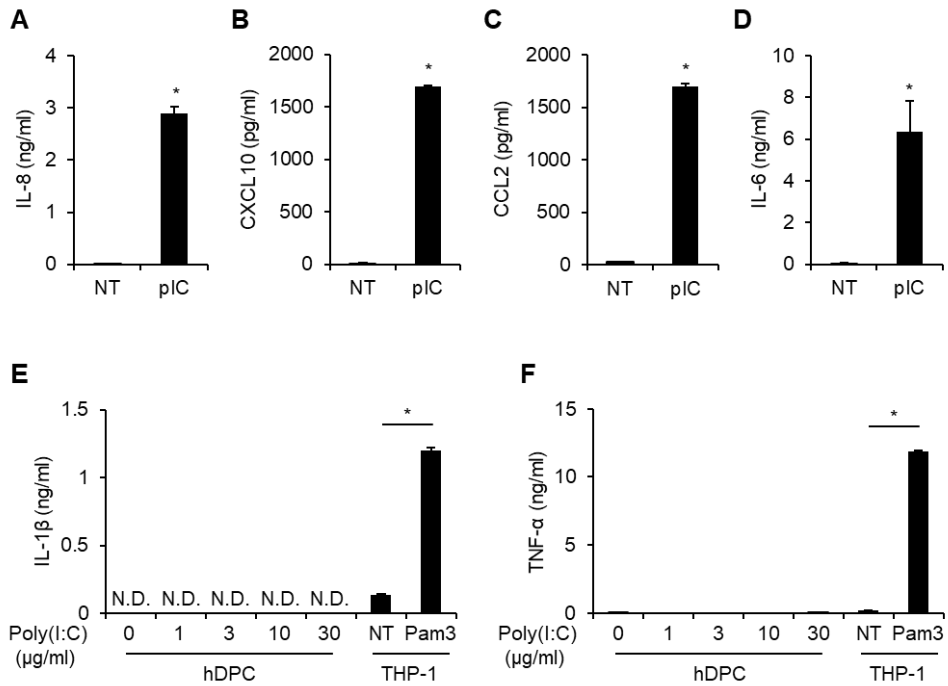

Figure 6. Poly(I:C) induces secretion of pro-inflammatory cytokines and chemokines but not IL-1β and TNF-α in human DPCs. (A-D) Human DPCs (1×10^5 cells/ml) were stimulated with 10 μg/ml of poly(I:C) for 24 h. The culture supernatants were collected and the concentrations of IL-8, CXCL10, CCL2 and IL-6 were determined by ELISA. (E-F) The concentrations of IL-1β and TNF-α were determined by ELISA. PMA-differentiated THP-1 cells were used as positive control for assay. N.D. denotes not detected. NT denotes non-treatment and pIC denotes poly(I:C). The results shown are representative of triplicated experiments. All results are expressed as mean ± SD of triplicate samples. Asterisks (*) indicate statistically significant differences ($P < 0.05$) compared with appropriate controls.

3.3. Poly(I:C) induces IL-8 secretion in human DPCs in time- and dose-dependent manner

To determine the immunostimulatory effect of TLR3 ligand, human DPCs from three donors were stimulated with poly(I:C). All human DPCs from three donors highly expressed *IL-8* mRNA after poly(I:C) treatment (Figure 7A-C). Furthermore, all human DPCs from each donor produced IL-8 to the culture supernatant after poly(I:C) treatment (Figure 7D-F). These results suggest that representative viral TLR3 ligand, poly(I:C) remarkably induces IL-8 production in human DPCs.

To examine the poly(I:C)-induced IL-8 expression kinetics, human DPCs were stimulated with poly(I:C) at indicated concentrations and times. The mRNA expression of IL-8 was significantly increased and protein level of IL-8 was also increased in a dose-dependent manner (Figure 8A-B). Time course analysis showed that IL-8 mRNA expression and protein production by 10 μg/ml of poly(I:C) stimulation were increased in a time-dependent manner (Figure 8C-D). The mRNA expression of IL-8 was already increased after 1 h of poly(I:C) treatment and reached a plateau after 12 h of poly(I:C) treatment.

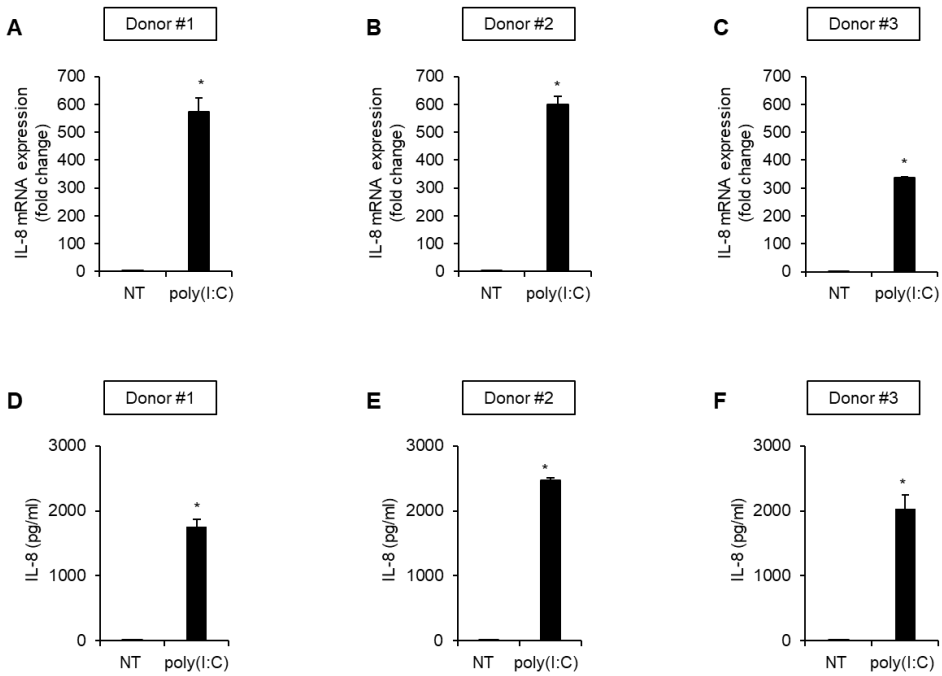

Figure 7. Poly(I:C) induces IL-8 secretion in human DPCs. (A-C) Human DPCs (1×10^5 cells/ml) were stimulated with 10 μg/ml of poly(I:C) for 3 h. mRNA expression of IL-8 was determined by real-time PCR. (D-F) Human DPCs were stimulated with 10 μg/ml of poly(I:C) for 24 h. The culture supernatants were collected and the concentration of IL-8 was determined by ELISA. NT denotes non-treatment. The results shown are representative of triplicated experiments. All results are expressed as mean ± SD of triplicate samples. Asterisks (*) indicate statistically significant differences ($P < 0.05$) compared with appropriate controls.

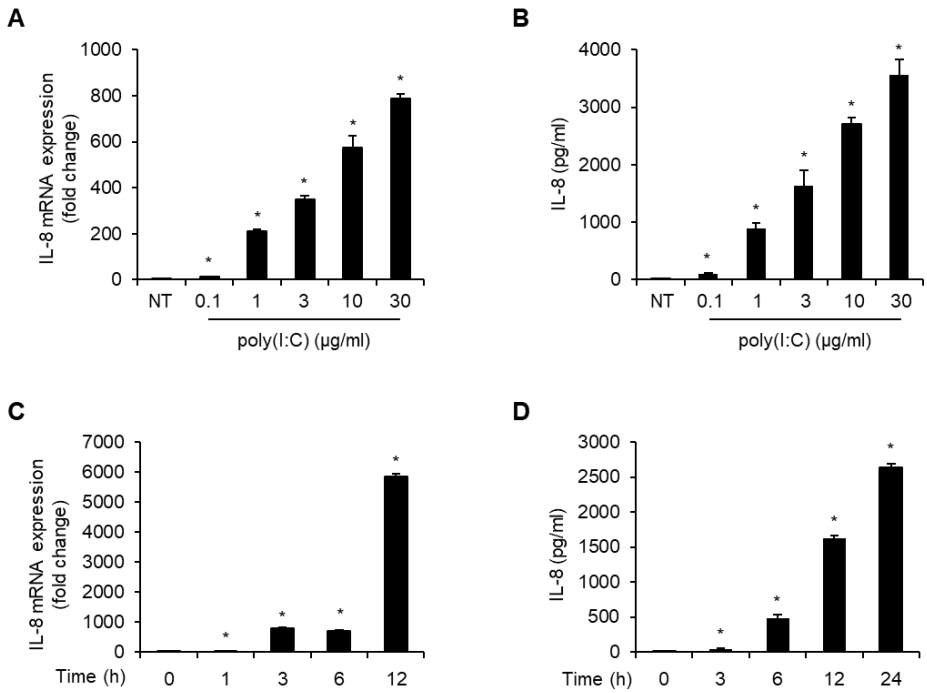

Figure 8. Poly(I:C) induces IL-8 secretion in human DPCs in time- and dose-dependent manner. (A) Human DPCs (1×10^5 cells/ml) were stimulated with indicated concentrations of poly(I:C) for 3 h. mRNA expression of IL-8 was determined by real-time PCR. (B) Human DPCs were stimulated with indicated concentrations of poly(I:C) for 24 h. The culture supernatants were collected and the concentration of IL-8 was measured by ELISA. (C) Human DPCs were incubated with 10 μg/ml of poly(I:C) for indicated times. mRNA expression of IL-8 was determined by real-time PCR. (D) Human DPCs were incubated with 10 μg/ml of poly(I:C) for indicated times. The culture supernatants were collected and the concentration of IL-8 was measured by ELISA. NT denotes non-treatment. The results shown are representative of triplicated experiments. All results are expressed as mean ± SD of triplicate samples. Asterisks (*) indicate statistically significant differences ($P < 0.05$) compared with appropriate controls.

3.4. Poly(I:C) induces pro-inflammatory cytokine productions through TLR3

To determine whether TLR3 signaling is involved in poly(I:C)-induced inflammatory cytokine production, human DPCs were transfected with human TLR3 specific siRNA. The mRNA expression of TLR3 was reduced by 71% in the cells treated with TLR3 siRNA compared to non-targeting siRNA as shown in Figure 9A. Human DPCs transfected with TLR3 siRNA showed decreased mRNA expressions of IL-8 and IL-6 after poly(I:C) stimulation compared with the cells transfected with non-targeting siRNA (Figure 9B-C). In addition, TLR3 siRNA-transfected human DPCs showed decreased IL-8 secretion in response to poly(I:C) (Figure 9D). These results suggest that TLR3 signaling is required for inflammatory cytokine production induced by poly(I:C).

It is well known that cytoplasmic dsRNA is recognized by RIG-1 and MDA5, and then activate transcriptional factors to induce type I IFN and pro-inflammatory cytokines. Therefore, the expression levels of intracellular dsRNA sensors, TLR3, RIG-1 and MDA5, were examined by real-time RT-PCR in the presence of poly(I:C) at the indicated times. The mRNA expression TLR3 increased after poly(I:C) treatment (Figure 10A). Also, the mRNA expression of *DDX58* (RIG-1) and *IFIH1* (MDA5) increased in the presence of poly(I:C) (Figure 10B-C). These results suggest that pro-inflammatory cytokine induction resulting from poly(I:C) stimulation is dependent on TLR3. Moreover, RIG-1 and MDA5 may contribute to cytokine induction.

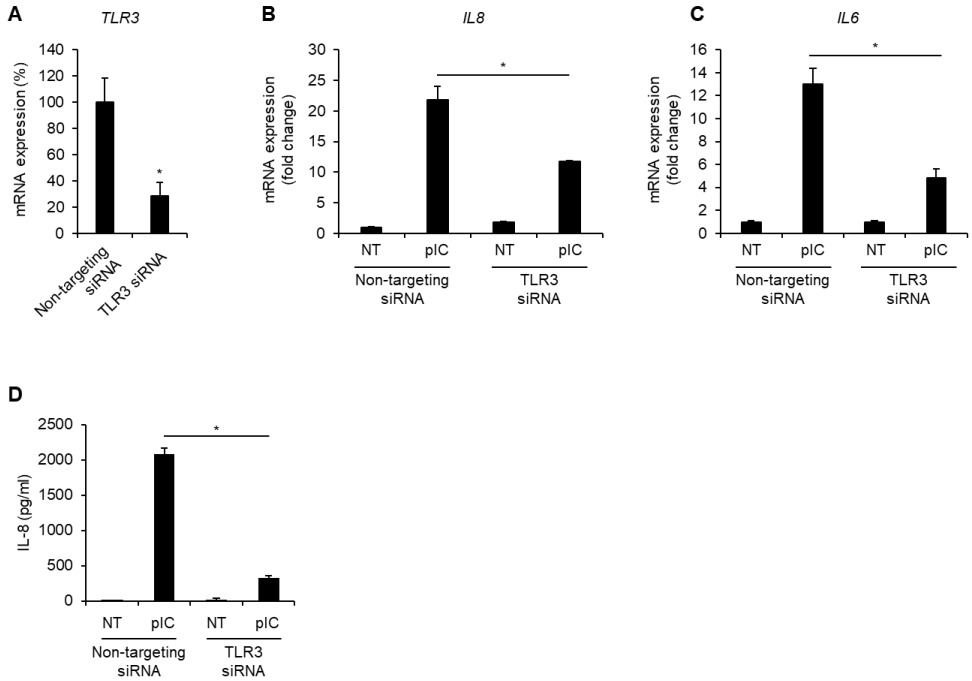

Figure 9. Poly(I:C)-induced pro-inflammatory cytokine production is dependent on TLR3. (A) Human DPCs (1×10^5 cells/ml) were transfected with TLR3 siRNA or non-targeting siRNA pool for 24 h. mRNA expression of TLR3 was determined by real-time RT-PCR. (B-C) Human DPCs (1×10^5 cells/ml) were stimulated with 10 μg/ml of poly(I:C) for 3 h followed by transfection with TLR3 siRNA or non-targeting siRNA pool for 24 h. mRNA expressions of IL-8 and IL-6 were determined by real-time PCR. (D) The transfected human DPCs (1×10^5 cells/ml) were stimulated with 10 μg/ml of poly(I:C) for 24 h. NT denotes non-treatment and pIC denotes poly(I:C). The culture supernatants were collected and the concentrations of IL-8 were determined by ELISA. All results are expressed as mean ± SD of triplicate samples. Asterisks (*) indicate statistically significant differences ($P < 0.05$) compared with appropriate controls.

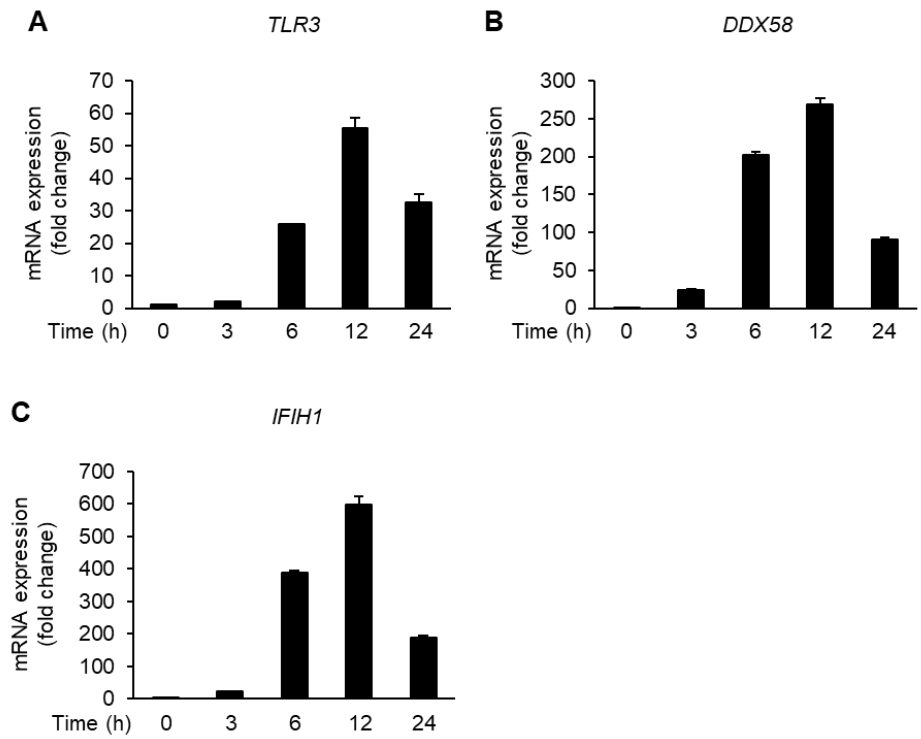

Figure 10. Poly(I:C) induces gene expression of RIG-1 and MDA5 in human DPCs. (A-C) Human DPCs (1×10^5 cells/ml) were stimulated with 10 μg/ml of poly(I:C) for indicated times. Total RNA was isolated from human DPCs. mRNA expressions of TLR3, DDX58 and IFIH1 were determined by real-time PCR. NT denotes non-treatment and pIC denotes poly(I:C). The results shown are representative of triplicated experiments. All results are expressed as mean ± SD of triplicate samples. Asterisks (*) indicate statistically significant differences ($P < 0.05$) compared with appropriate controls.

3.5. MAP kinase signaling pathway is involved in poly(I:C)-induced IL-8 secretion of human DPCs

It was reported that MAP kinase signaling is necessary for cytokine production through TLR activation [45, 46]. Therefore, to examine whether MAP kinase is required for IL-8 induction following TLR3 activation, human DPCs were pretreated with MAP kinase inhibitors at the indicated concentrations and then stimulated with poly(I:C). There was no observed cytotoxicity of the inhibitors at the concentrations used. The inhibition of p38 by SB203580 and SB202190 significantly reduced poly(I:C)-induced IL-8 secretion from human DPCs following poly(I:C) stimulation in a dose-dependent manner (Figure 11A-B). SB203580 and SB202190 decreased IL-8 secretion in human DPCs from a concentration of 1 μM and potently decreased at a concentration of 40 μM. Also, the inhibition of ERK by PD90859 and ERK inhibitor II decreased poly(I:C)-induced IL-8 induction from human DPCs in the presence of poly(I:C) in a dose-dependent manner (Figure 11C-D). PD90859 significantly decreased IL-8 secretion in human DPCs at concentrations of 10 μM and 40 μM. ERK inhibitor II significantly decreased IL-8 secretion in human DPCs from at a concentration of 2.5 μM. In addition, the inhibition of JNK by SP600125 and JNK inhibitor II also significantly decreased poly(I:C)-induced IL-8 secretion in a dose-dependent manner (Figure 11E-F). These results indicated that MAP kinase signaling pathway is essential for poly(I:C)-induced IL-8 production.

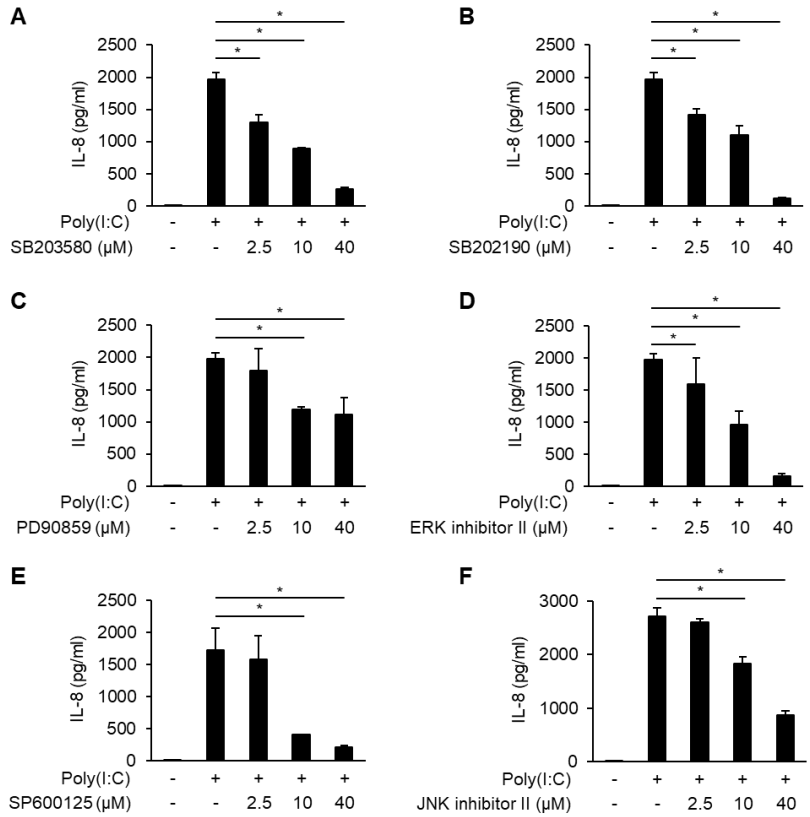

Figure 11. MAP kinase signaling pathway is involved in poly(I:C)-induced IL-8 secretion of human DPCs. (A-B) Human DPCs (1×10^5 cells/ml) were stimulated with 10 μg/ml of poly(I:C) following pre-treatment with p38 inhibitors, SB203580 and SB202190, for 1 h. The culture supernatants were collected and the concentrations of IL-8 were determined by ELISA. (C-D) Human DPCs were stimulated with 10 μg/ml of poly(I:C) following pre-treatment of ERK inhibitors, PD90859 and ERK inhibitor II, for 1 h. (E-F) Human DPCs were stimulated with 10 μg/ml of poly(I:C) following pre-treatment of JNK inhibitors, SP600125 and JNK inhibitor II, for 1 h. The culture supernatants were collected and the concentrations of IL-8 were determined by ELISA. All results are expressed as mean ± SD of triplicate samples. Asterisks (*) indicate statistically significant differences ($P < 0.05$) compared with appropriate controls.

3.6. NF-κB and C/EBPβ are essential transcriptional factors for poly(I:C)-induced IL-8 production in human DPCs

It is reported that transcriptional factor AP-1, C/EBPβ and NF-κB are essential for *IL8* expression and binding sites of these transcriptional factors are located at *IL8* -133-bp promoter region [47]. To investigate which transcriptional factors are involved in regulation of *IL8* expression in human DPCs, IL-8 promoter activity was analyzed by luciferase reporter gene assay. Human DPCs were transiently transfected with a luciferase reporter plasmid containing human IL-8 promoter (-132/+42)/pGL3 WT, mut AP-1, mut C/EBPβ and mut NF-κB constructions which were mutated on each transcriptional factor binding site (Figure 12A). As expected, IL-8 promoter WT transfected human DPCs showed increase in IL-8 promoter activity in the presence of poly(I:C) and IL-8 promoter activity was suppressed when transfected with mut C/EBPβ and mut NF-κB compared with WT (Figure 12B). In a similar context, chemical inhibitor of NF-κB, BAY11-7082, inhibited IL-8 secretion from human DPCs following poly(I:C) stimulation (Figure 12C). Therefore, these results indicate that NF-κB and C/EBPβ are essential transcriptional factors for poly(I:C)-induced *IL8* expression in human DPCs.

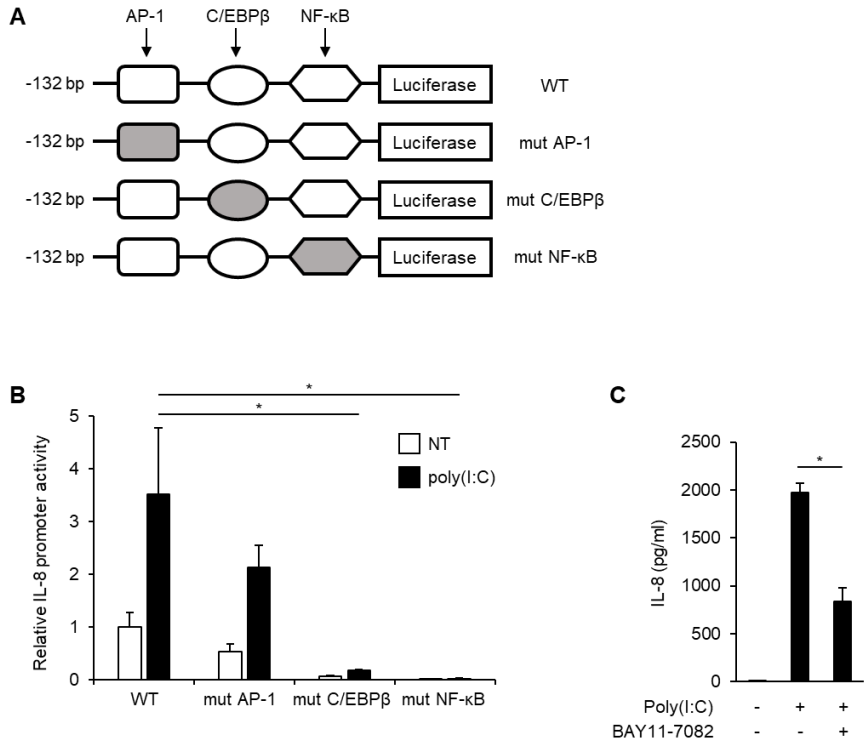

Figure 12. NF-κB and C/EBPβ are essential transcriptional factors for poly(I:C)-induced IL-8 production. (A) Human IL-8 promoter (-132/+42)/pGL3 construction. WT is intact construction. mut AP-1, mut C/EBPβ and mut NF-κB are mutated on each transcriptional factor binding site. (B) Human DPCs (1×10^5 cells/ml) were transiently transfected with human IL-8 promoter (-132/+42)/pGL3 WT, mut AP-1, mut C/EBPβ and mut NF-κB mutated on each transcriptional factor binding site. The transfected cells were then stimulated with 10 μg/ml of poly(I:C) for 24 h and luciferase activity was normalized by *Renilla* luciferase activity. (C) Human DPCs (1×10^5 cells/ml) were pre-treated with BAY11-7082 for 1 h and stimulated with 10 μg/ml of poly(I:C) for 24 h. The culture supernatants were collected and the concentrations of IL-8 were determined by ELISA. All results are expressed as mean ± SD of triplicate samples. Asterisks (*) indicate statistically significant differences ($P < 0.05$) compared with appropriate controls.

3.7. SCFAs down-regulate poly(I:C)-induced IL-8 production in human DPCs

SCFAs are metabolites produced by commensal microbiota through fermentation of dietary fiber; acetate, propionate and butyrate exist predominantly [48]. Recently, it was reported that periodontal pathogens also produce SCFAs as metabolites and the levels of SCFAs are significantly higher in the saliva of patients with severe periodontal disease [49]. In this point of view, I hypothesized SCFAs generated from periodontal pathogens may affect pulpal inflammation. To examine the effects of SCFAs on poly(I:C)-induced pulpal inflammation, human DPCs were treated with poly(I:C) in the presence or absence of SCFAs (acetate, propionate or butyrate). As a result, poly(I:C)-induced IL-8 production was significantly decreased in the presence of butyrate and propionate at concentration of 1 mM (Figure 13B-C). Acetate significantly inhibited poly(I:C)-induced IL-8 production at concentration of 30 mM (Figure 13A). These results suggest that in human DPCs, butyrate, propionate and acetate potently suppress poly(I:C)-induced IL-8 secretion in order.

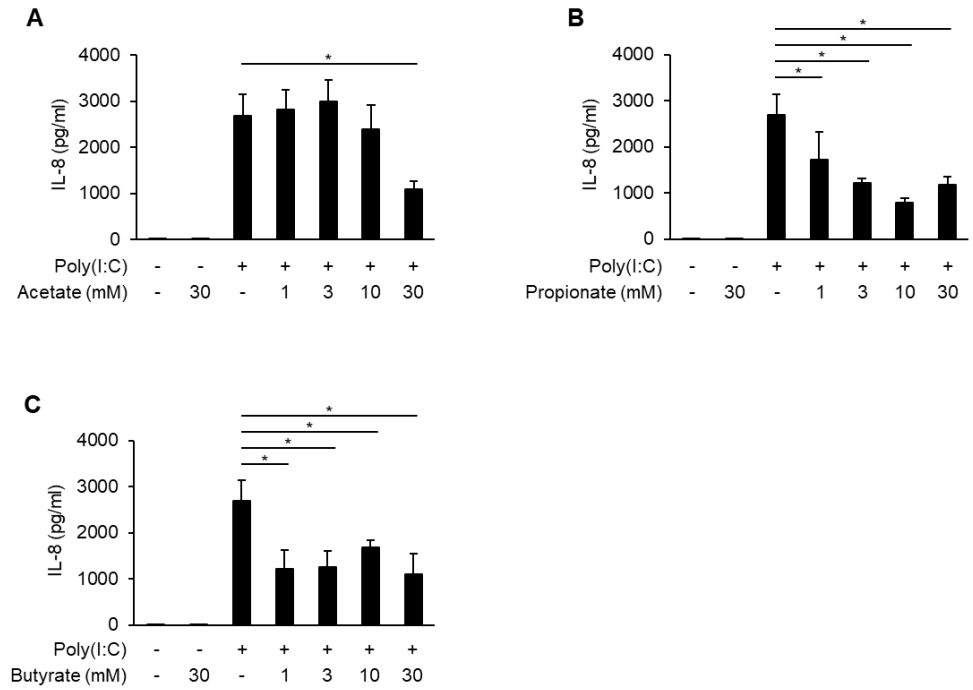

Figure 13. SCFAs down-regulate poly(I:C)-induced IL-8 production in human DPCs. (A) Human DPCs (1×10^5 cells/ml) were stimulated with 10 μg/ml of poly(I:C) in the presence or absence of acetate at indicated concentrations. (B) Human DPCs (1×10^5 cells/ml) were stimulated with 10 μg/ml of poly(I:C) in the presence or absence of propionate at indicated concentrations. (C) Human DPCs (1×10^5 cells/ml) were stimulated with 10 μg/ml of poly(I:C) in the presence or absence of butyrate at indicated concentrations. After 24 h incubation, the culture supernatants were collected and the concentrations of IL-8 were determined by ELISA. All results are expressed as mean ± SD of triplicate samples. Asterisks (*) indicate statistically significant differences ($P < 0.05$) compared with appropriate controls.

3.8. Lipopeptide and poly(I:C) synergistically enhance IL-8 production in human DPCs

There are numerous species of bacteria in the oral and it was reported that polymicrobial infection of periodontal pathogens resulted in cooperative effects on cytokine production from immune cells [50]. Indeed, bacteria can facilitate viral infection of oral fibroblasts by using their virulence factor such as LPS [51]. To investigate synergistic effect of poly(I:C) and bacterial component, human DPCs were stimulated with Pam2CSK4, LPS or flagellin in the presence or absence of poly(I:C). Poly(I:C) co-treatment with LPS or flagellin showed additive effect on IL-8 production. Interestingly, poly(I:C) co-treatment with Pam2CSK4 synergistically enhanced IL-8 secretion (Figure 14A). These results indicate that Pam2CSK4 potentiates poly(I:C)-induced IL-8 expression in human DPCs. To determine how Pam2CSK4 and poly(I:C) synergistically induce inflammatory cytokine, human DPCs were transfected with siRNA specific for TLR2 or TLR3. As a result, Pam2CSK and poly(I:C)-induced IL-8 secretion was significantly decreased by TLR3 knock down (Figure 14B).

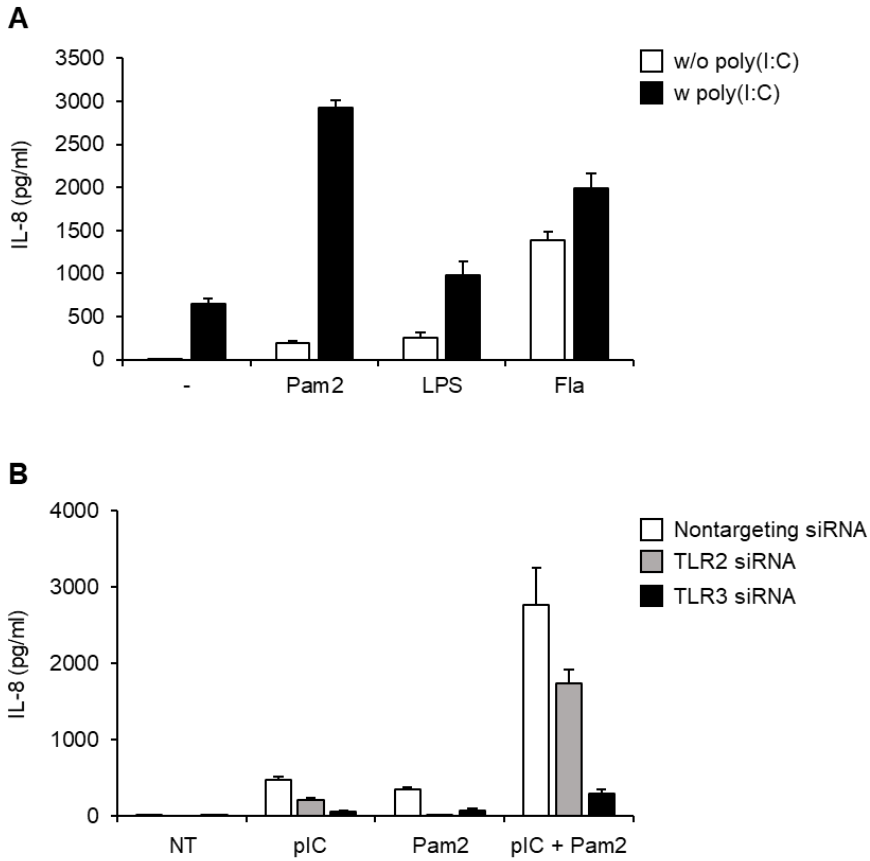

Figure 14. Pam2CSK4 and poly(I:C) synergistically enhance IL-8 production. (A) Human DPCs (1×10^5 cells/ml) were co-treated with 1 μg/ml of poly(I:C) and 1 μg/ml of Pam2CSK4, LPS or flagellin for 24 h. The culture supernatants were collected and the concentration of IL-8 was determined by ELISA. (B) Human DPCs (1×10^5 cells/ml) were transfected with TLR2 siRNA, TLR3 siRNA or non-targeting siRNA. The transfected cells were stimulated with indicated stimuli at concentration of 1 μg/ml for 24 h. The culture supernatants were collected and the concentrations of IL-8 were determined by ELISA. All results are expressed as mean ± SD of triplicate samples.

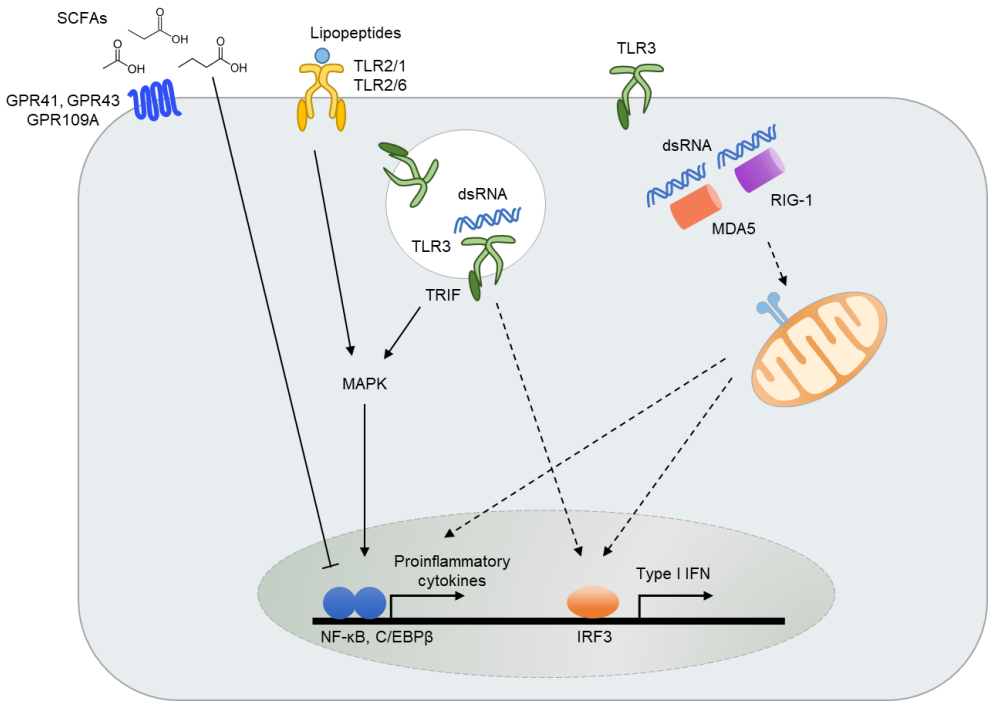

Figure 15. Schematic illustration of the proposed mechanism. TLR3 is mainly located in intracellular in human DPCs. dsRNA is recognized by TLR3 and activate MAP kinase signaling. Also, dsRNA could be recognized by cytoplasmic receptors, RIG-1 and MDA5. Subsequently, TLR3-TRIF and RIG-1/MDA5 signalings activate transcriptional factors. Then, activated transcriptional factors induce expression of pro-inflammatory cytokines and type I IFN, resulting in recruitment of innate and adaptive immune cells. Moreover, lipopeptide and poly(I:C) have synergistic effect on inflammatory cytokine induction by amplification of MAPK activation. In contrast, SCFAs down-regulate inflammatory cytokine induction via HDAC inhibition or GPCR activation. Solid lines mean action mechanism based on the results observed in the present study. Broken lines mean proposed action mechanism.

Chapter IV. Discussion

In this study, TLR expression profile of human DPCs was analyzed and the immunostimulatory properties of various TLR ligands in human DPCs were studied. Among 10 TLRs (TLR1-10), TLR3 was the most highly expressed in human DPCs. Poly(I:C), a representative viral TLR3 ligand, efficiently induced pro-inflammatory cytokines including IL-8 in human DPCs. MAP kinases (p38, EKR and JNK), C/EBPβ and NF-κB were involved in poly(I:C)-induced IL-8 expression in human DPCs. Furthermore, SCFAs down regulated poly(I:C)-induced IL-8 production and lipopeptide showed synergistic effect with poly(I:C) in IL-8 production in human DPCs. These results suggest that TLR3 might be essential receptors for pro-inflammatory responses against viral infections and bacteria-derived TLR2 ligand such as lipopeptide might affect virus-induced inflammatory responses in pulpal environment.

Human DPCs consistently expressed TLR3 and it was higher when compared with other TLRs. It is likely that consistent TLR3 expression in human DPCs might be essential for immune surveillance against viral infections. Accumulating studies reported that human DPCs expressed TLRs in different quantities according to inflammatory conditions. For instance, TLR2, TLR3, TLR4, TLR5 and TLR8 were up-regulated but other TLRs (TLR1, TLR6, TLR7, TLR9 and TLR10) were down-regulated in inflammatory conditions [52]. Indeed, Kaposi's sarcoma-associated herpesvirus can up-regulate TLR3 expression during viral infection in human monocytes [53]. Therefore, the expression of TLR3 in human DPCs seems to be

affected by oral viruses considering that Epstein-Barr virus was frequently founded in patients with irreversible pulpitis or apical periodontitis compared with healthy controls [54]. In another study of periodontal ligament cells, TLR3 expression was significantly higher than other TLRs and TLR3 was observed in endosomal compartment [55]. Concordantly, the mRNA expression of TLR3 increased after poly(I:C) treatment in this study, but further study is needed since there may be individual differences in TLR expression of human DPCs for several reasons such as oral microbiota and inflamed condition.

Poly(I:C) potently induced various chemokines including IL-8 in human DPCs. Similarly, it has been reported that IL-8 expression was increased in inflamed dental pulps [31]. In addition, poly(I:C) induced IL-8 expression in various cell types such as macrophages [56], intestinal epithelial cells [57], lung epithelial cells [58] and astrocytes [59]. IL-8 is important for initiating and maintaining various immune and inflammatory reactions [32]. Therefore, poly(I:C)-induced IL-8 production could exacerbate oral diseases such as pulpitis and apical periodontitis through induction of inflammatory response from human DPCs. Furthermore, poly(I:C) induced not only IL-8 but also other cytokines including IFN-β, IL-6, CCL2 and CXCL10. These results are consistent with previous report that poly(I:C) potentiated IL-6 secretion in human DPCs [41]. Poly(I:C) seems to be a potent chemokine inducer in human DPCs. Although further studies are needed to identify the role of poly(I:C)-induced cytokines during viral infection, it might contribute to infiltration and activation of other immune cells for amplification of inflammatory responses in pulp tissues.

In addition, mRNA expression of IL-1β and TNF-α were significantly increased by poly(I:C) in human DPCs, however the protein productions were negligible. These results are concordant with a previous report that IL-1β and TNF-α were not detected from human DPCs culture supernatants [60]. This phenomenon that negligible secretion of IL-1β and TNF-α seems to be an inherent reaction to poly(I:C) in human DPCs while human DPCs expressed NLRP3 and AIM2 and secreted IL-1β via AIM2 inflammasome activation in response to dsDNA [61, 62]. It suggests that human DPCs induce chemokine expression against viral dsRNA to initiate innate and adaptive immune responses, however, human DPCs rarely produce IL-1β or TNF-α that leads to chronic inflammatory conditions without excessive inflammation in pulp tissue.

The current study demonstrated that MAP kinases (p38, ERK and JNK) were involved in IL-8 expression by poly(I:C) stimulation in human DPCs. MAP kinases including p38, ERK and JNK are generally activated in response to TLR signaling [63] and regulate pro-inflammatory cytokine expressions in various cell types [42, 46, 64]. Indeed, TLR3-mediated activation of p38 induces stabilization of IFN-β transcripts [65]. It suggests that activation of p38, ERK and JNK might stimulate IL-8 production and contribute to IFN-β expression in response to dsRNA. In addition, C/EBPβ and NF-κB were essential for poly(I:C)-induced IL-8 expression in human DPCs. The expression of IL-8 is mainly regulated by AP-1, C/EBPβ and NF-κB [66]. Among these transcriptional factors, C/EBPβ and NF-κB synergistically enhance IL-

8 expression [67] . It is consistent with the results shown in Figure 12B and indicate that C/EBPβ and NF-κB are essential for poly(I:C)-induced IL-8 expression in human DPCs and both of them must be required for potent IL-8 expression.

In this study, it was demonstrated that poly(I:C)-induced cytokine productions in human DPCs were mediated by intracellular compartments such as TLR3, RIG-1 and MDA5. Extracellular dsRNA is internalized by clathrin-dependent endocytosis and recognized by endosomal TLR3 [16, 68]. Also, RIG-1 and MDA5 are crucial for the recognition of cytoplasmic dsRNA [19]. In the current study, real-time RT-PCR analysis showed that the expression kinetics of IL-8 showed two-step induction at 3 h and 12 h after poly(I:C) stimulation. This two-step induction of IL-8 gene expression may be caused by distinct signals which are TLR3-mediated signaling and RIG-1/MDA5-mediated signaling. SIDT2, an endosomal transmembrane protein, transports dsRNA from endosome to the cytoplasm [69]. There were no differences in HSV-induced IFN-β production between wild-type mice and SIDT2 knockout mice at 8 h post infection, but IFN-β production in SIDT2 knockout mice was lower than wild-type mice after 16 h post infection. Therefore, it is likely that endosomal TLR3 mediate early immune responses against dsRNA, and then RIG-1 and MDA5 recognize dsRNA which transported to cytoplasm via SIDT2.

Accumulating studies have reported that viruses interact with bacteria or bacterial products, and their interactions enhance coinfection rates and affect the host immune responses [70, 71]. Superinfections can be caused by secondary infections following

viral infections [72]. In the present study, lipopeptide, a synthetic analog of bacterial lipoproteins, and poly(I:C) showed synergistic effect on IL-8 expression in human DPCs. Similarly, it has been reported that co-treatment of Pam3CSK4 and poly(I:C) enhances inflammatory cytokine production in B cells [73], macrophages [74], and airway epithelial cells [75], suggesting that it seems to be a general phenomenon. Even though further studies are needed to identify the detailed molecular mechanisms of viral and bacterial coinfection, both TLR2 and TLR3 might be important innate immune receptors that initiate pro-inflammatory responses in pulp tissues.

Three major SCFAs (acetate, propionate and butyrate) reduced poly(I:C)-induced IL-8 secretion in human DPCs. It has been reported that the concentration of SCFAs are higher in the saliva and gingival crevicular fluid of patients with severe periodontitis compared with healthy individuals [49, 76]. SCFAs can directly activate G protein-coupled receptor (GPCR), such as GPR41, GPR43 and GPR109a [77, 78] and epigenetically regulate immune cell functions by histone deacetylase (HDAC) inhibition. Our previous reports demonstrated that SCFAs reduced nitric oxide production mediated by Staphylococcal lipoprotein via HDAC inhibition [79]. In this study, among SCFAs, butyrate most potently suppressed poly(I:C)-induced IL-8 production in human DPCs. These results are concordant with other studies showed that SCFAs modulate anti-inflammatory function of macrophages and neutrophils [80, 81]. In contrast, several studies have been reported that butyrate increases pro-inflammatory cytokines. It is demonstrated that butyrate increased CCL20 secretion from human intestinal epithelial cells and gingival epithelial cells

[82, 83] and potentiated LPS-induced IL-1β secretion from macrophages [84]. The anti-inflammatory functions of SCFAs are still controversial, thus further study is needed on how SCFAs regulate immune response against poly(I:C) in human DPCs.

Human DPCs can be exposed to oral microorganisms through dentinal tubule exposure. During microbial infection through dentinal tubule exposure, odontoblasts can be exposed to microorganisms before human DPCs due to the fact that odontoblasts are located in the outermost layer of pulp and extend odontoblastic processes to dentinal tubules. Interestingly, recent studies provide clues that virus can affect human DPCs via extracellular vesicles (EVs) released from infected host cells [85]. EVs generated from infected cells can contain viral proteins and even infectious viral genomes [86, 87]. In addition, EVs are nanoscale particles 50-100 nm in size, so it is possible to reach human DPCs by passing dentinal tubules. However, the effect of EVs on oral environment is limited, therefore, further study is needed on salivary EVs and viral oral disease.

Viruses are closely related to various oral diseases including pulpitis, apical periodontitis, and gingivitis [7]. The current study showed that TLR3 is most highly expressed on human DPCs among TLRs 1-10 and poly(I:C) which is a synthetic analog of viral dsRNA, is a potent immune stimulator which activates TLR3 signaling pathway. In addition, TLR2 ligand, such as Pam2CSK4, potentiates poly(I:C)-induced inflammatory responses in human DPCs. On the other hand, SCFAs, microbial metabolites, down-regulate poly(I:C)-induced chemokine

production. This study will provide new insight of viral infection by microorganisms and viral-bacterial interaction in pulpal microenvironment.

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국문초록

사람치수세포에서 Toll 유사 수용체 3을 통한 케모카인 유도

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1. 목적

치수강에 존재하는 치수세포(dental pulp cell)는 중간엽줄기세포의 특성이 있으며 상아아세포, 지방세포, 연골세포, 조골세포, 신경세포 등으로 분화할 수 있다. 치수세포는 치아우식증, 치아 손상, 상아세관을 통해 구강 내 미생물에 노출될 수 있고 미생물-연관 분자 패턴(microbe-associated molecular pattern)에 의한 염증반응이 일어나 치수염으로 이어질 수 있다. 이때 IL-1, IL-6, IL-8, TNF-α, MMP-9을 포함한 염증 매개물이 치수염을 악화시킬 수 있다. 구강에는 다양한 미생물이 존재하고 이들은 여러 감염성 구강 질환과 밀접한 연관이 있다. 치수 조직이 구강내 미생물에 노출되었을 때 몇몇의 구강 바이러스는 치수세포의 면역반응을 유도할 수 있다. 그러나 치수세포와 미생물과의 상호작용 및 이에 대한 면역학적 특성에 대한 연구는 부족한 실정이다. 치수세포는 감염 및 손상 후 치수재생에

중요한 역할을 하기 때문에 병인 기전 연구 및 치료제 개발을 위해선 치수세포의 면역학적 특성에 관한 연구가 필요하다. 이와 같은 연구를 통해 미생물 유래 인자와 치수세포의 상호작용을 분자적 수준에서의 이해를 돕고 병인 기전의 심층적인 이해를 도울 수 있다. 따라서 본 연구에서는 구강 내 바이러스 및 세균에 의한 구강질환 예방 및 치료방법 개발에 중요한 기초지식을 제공하고자 한다.

2. 방법

사람치수세포를 이용한 실험은 서울대학교 치의학대학원 연구윤리심의위원회의 승인을 받아 수행하였다(S-C20200023). 사람치수에서 치수세포를 분리하여 일차 배양 하였다. 치수세포의 미생물 유래 물질을 인지할 수 있는 수용체의 발현 양상을 확인하기 위해 real-time RT-PCR 방법을 이용하여 Toll 유사 수용체의 mRNA 발현 수준을 확인하였다. Poly(I:C) 처리 시 치수세포에서 분비되는 염증반응에 관여하는 싸이토카인 및 케모카인의 발현 양상을 확인하기 위해 real-time RT-PCR을 이용하여 IL-8, CXCL10, CCL2, CCL5, CCL20, IFN-β, IL1-β, IL-6, TNF-α 의 mRNA 발현을 확인하였고, ELISA를 이용하여 IL-8, CXCL10, CCL2, IL-1β, IL-6, TNF-α를 단백질 수준에서 확인하였다. 또한, TLR3 siRNA를 transfection한 뒤 poly(I:C)를 처리하여 IL-8 발현에 차이가 생기는지 real-time RT-PCR, ELISA를 이용하여 mRNA 및 단백질 수준에서 확인하였다. 신호전달경로를 확인하기 위해 MAP kinase 억제제를 농도별로 전 처리 후 poly(I:C)를 처리하고 ELISA로 IL-8 분비에 차이가 있는지 확인하였다. 인간 IL-8 유전자 프로모터의 전사 조절 인사 결합부위에 돌연변이를 가진 리포터 유전자를 transfection 한 뒤 luciferase assay를 하여 poly(I:C) 처리 시 유도되는 IL-8 분비에 주요한 영향을 미치는 전사 조절 인자를 분석하였다.

3. 결과

사람치수세포에서 미생물 유래 물질을 인지할 수 있는 수용체의 발현을 확인하기 위해 Toll 유사 수용체들(TLRs)의 mRNA 발현 수준을 비교하였을 때 TLR3의 발현량이 상대적으로 가장 높고 TLR6, TLR5, TLR1 또한 비교적 높게 발현하고 있음을 확인하였다. 이에 따라, TLR3의 리간드이자 바이러스에서 유래하는 dsRNA의 모사물질인 poly(I:C)를 처리하였을 때 IL-8의 mRNA 및 단백질 수준에서 모두 발현이 유의하게 증가하였다. 또한, 이는 poly(I:C)의 농도 및 처리시간에 의존적으로 증가하였다. Poly(I:C)에 대한 치수세포의 면역학적 특성을 확인하기 위해 여러 싸이토카인과 케모카인의 발현을 확인해 보았을 때, poly(I:C)에 의해 IL-8 뿐만 아니라 CXCL10, CCL2, CCL5, CCL20, IFN-β, IL-6의 발현도 유의하게 증가하였다. 다만, 치수세포에서 염증성 싸이토카인인 IL-1β, TNF-α는 분비되지 않았다. 치수세포가 poly(I:C)를 인지하는 것이 TLR3 의존적인지 확인하기 위해 TLR3 siRNA를 transfection 하였다. 치수세포에서 TLR3가 knock down 되었을 때는 대조군과 비교해 poly(I:C)에 대한 IL-8 생성이 유의하게 감소하였다. 그리고 세포질에 존재하는 dsRNA 수용체인 MDA5와 RIG-1의 mRNA 발현이 증가함을 확인하였다. 신호전달경로 확인을 위해 p38 억제제(SB203580, SB202190), ERK 억제제(PD90859, ERK inhibitor II), JNK 억제제를 전처리 하였을 때, 전처리한 억제제의 농도에 의존적으로 poly(I:C)에 의한 IL-8 생성이 감소하는 것을 통해 MAP kinase signaling pathway가 관여함을 확인하였다. 또한, 인간 IL-8 프로모터의 전사인자 결합부위(AP-1, NF-κB, C/EBPB)에 돌연변이를 가진 리포터 유전자를 transfection 하여 luciferase reporter gene assay 한 결과, NF-κB, C/EBPB가 poly(I:C)에 의한 IL-8 발현에 주요한 전사인자임을 확인하였다. 바이러스와 박테리아의 상호작용이 구강질환에 영향을 미칠 수 있다는 것을 고려하여 poly(I:C)와 박테리아 유래 물질을 병용처리하여 면역반응에 어떠한 영향을 미치는지 확인하고자 하였다. 그 결과 poly(I:C) 와 lipopeptide가 poly(I:C)에 의한 IL-8 발현에 상승효과 있음을 확인하였다.

구강 및 소화관의 상주균이 식이섬유를 발효하여 대사물질로 단쇄지방산인 acetate, propionate, butyrate가 생성되는데, 이러한 미생물 유래 물질을 poly(I:C)와 병용처리 하였을 때 IL-8의 발현을 감소시키는 효과가 있음을 확인하였다.

4. 결론

위의 결과들을 종합하여 보면, 치수세포는 dsRNA를 인지할 수 있는 TLR3를 높은 수준으로 발현하고 있어 구강 내 바이러스에 대한 강한 면역반응을 유도할 수 있음을 시사한다. 치수세포는 poly(I:C)에 대한 반응으로 IL-8을 비롯하여 IL-6, IFN-β, CCL2, CXCL10, CCL5, CCL20을 분비함으로써 바이러스에 대한 면역반응을 개시한다. 치수세포에서 poly(I:C)에 의한 이러한 면역반응은 TLR3를 통해 인지되어 MAP kinase signaling pathway를 경유하고 NF-κB, C/EBPβ 전사인자를 활성화시킴으로써 일어난다. 더불어 상주균이 대사물질로 분비하는 단쇄지방산인 acetate, propionate, butyrate가 poly(I:C)에 의한 싸이토카인 분비를 완화하여 면역반응을 조절할 수 있다.

주요어: 치수세포, double-stranded RNA, poly(I:C), TLR3, IL-8
학번: 2018-22226

감사의 글

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